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ORIGINAL PAPER

Integrative redescription of a common Arctic water bear *Pilatobius recamieri* (Richters, 1911)

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Abstract Tardigrada are a group of microscopic metazoans that inhabit a variety of ecosystems throughout the world, including polar regions, where they are a constant element of microfauna with densities exceeding hundreds of individuals per gram of dry plant material. However, despite a long history of research and their ubiquity in tundra ecosystems, the majority of tardigrade species have limited and outdated diagnoses. One such example is *Pilatobius recamieri*, a common tardigrade that is widely distributed in the Arctic. The aim of this study is to redescribe this species using new material from the type locality and tools of integrative taxonomy, viz. by combining classical imaging and morphometry by light microscopy and scanning electron microscopy imaging with DNA sequencing of four markers with various mutation rates: three nuclear (18S rRNA, 28S rRNA, and ITS-2) and one mitochondrial (COI). The sequences of the three latter markers are also the first to be presented for the genus *Pilatobius*. This study therefore provides the first necessary step towards the verification of the geographic range of *P. recamieri*, which is currently assumed to be very broad. A detailed comparison of *P. recamieri* with *Pilatobius secchii* (Bertolani and Rebecchi, 1996) from Italy revealed no morphological or morphometric differences between the two species, thus we designate *P. secchii* as a *nomen inquirendum* until molecular data for the taxon become available. Finally, we propose to replace the term "lunula" in the superfamilies Hypsibioidea and Isohypsibioidea with the more appropriate "pseudolunula" to differentiate it from the true lunula in other parachelans.

Keywords Biodiversity · Hypsibiidae · Microfauna · *Pilatobius secchii* nom. inq. · Svalbard · Tardigrada

Introduction

The tardigrades, also known as water bears, are common micrometazoans, usually less than 1 mm in length. They are distributed across the globe, inhabiting a great majority of terrestrial (soil, plants, and leaf litter), freshwater, and marine ecosystems (plants, coastal and deep-oceanic sediments), from tropical and temperate regions to the highest mountain peaks, glaciers, and polar deserts (e.g., Nelson et al. 2015). They are widely known for their cryptobiotic capabilities, thanks to which they can withstand extreme conditions such as low and high temperatures, desiccation, and high ultraviolet radiation doses (e.g. Guidetti et al. 2012). These adaptations allow tardigrades to dwell in harsh environmental conditions, including those shaping polar ecosystems (McInnes and Ellis-Evans 1990; Altiero et al. 2015; Zawierucha et al. 2015). The Svalbard Archipelago is located in the European part of the Arctic and is one of the best investigated polar areas in terms of tardigrade fauna. Studies of Svalbardian tardigrades began in the late 19th century (Scourfield 1897) and have been

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continued by a number of researchers up to the present day (e.g., Węglarska 1965; Dastych 1985; Maucci 1996; Tumanov 2007; Zawierucha et al. 2015, 2016a).

Because of tardigrade ubiquity, interest in the ecology of Arctic tardigrades has increased during the last decade (Coulson and Midgley 2012; Johansson et al. 2013; Zawierucha et al. 2016a, b). Since species are considered the basic units of ecosystems (e.g., Callaghan et al. 2004), correct species identification is crucial to ecological studies. This is especially important in the face of the impact of climate change on Arctic biota and to understand the biogeographical patterns of life affected by these shifts, as some studies suggest that species alter their distributions rather than evolve in response to environmental changes influenced by global warming (Callaghan et al. 2004; Hodkinson 2013). However, tardigrade taxonomy is based largely on morphological and morphometric traits (e.g., see Michalczyk and Kaczmarek 2013; Kosztyła et al. 2016), and many species descriptions are incomplete and grossly outdated, with molecular data being available for only a small fraction of species (e.g., see Bertolani et al. 2014). One such species with a poor diagnosis is *Pilatobius recamieri* (Richters, 1911), a limnoterrestrial eutardigrade described from and frequently found in the Svalbard Archipelago (Richters 1911; Zawierucha et al. 2016a, b). The species was subsequently reported from other Arctic localities as well as from Europe, Asia, and North and South America (Ramazzotti and Maucci 1983). However, given the limited original description, the exact geographic range of the species cannot be confidently outlined. For example, a recent study by Gąsiorek et al. (2016) on *Mesocrista spitzbergensis* (Richters, 1903), a tardigrade originally described from Spitsbergen and subsequently reported from numerous Holarctic localities, suggested that the species might have a much more limited geographic range than was previously assumed. Detailed morphological and molecular analyses showed that specimens collected from several European localities represented, in fact, a new species, *Mesocrista revelata* Gąsiorek et al., 2016. Thus, it is plausible that a modern redescription of *P. recamieri* could also reveal more than one species hiding under a single name, thereby limiting the geographic range of *P. recamieri*.

In this paper, we integratively redescribe *P. recamieri* from its *terra typica* in the Svalbard Archipelago. In addition to classic morphometry and imaging by light microscopy, we also reveal fine morphological traits by using scanning electron microscopy and provide sequences for three nuclear and one mitochondrial DNA marker. We also analyzed paratypes of *Pilatobius secchii* (Bertolani and Rebecchi, 1996), a species that is morphologically most similar to *P. recamieri*. It is hoped that this study, by setting a reference point for future records of *P. recamieri*, will enable a verification of the true geographic range of the species.

Materials and methods

Samples and specimens

We analyzed a total of 48 individuals of *P. recamieri* from a neotypic population in a sample collected from *terra typica* by the second author on the 29th July 2013 (79°50′N, 11°18′E; 40 m above sea level; Fuglesangen, Svalbard, Norway; tundra, moss from rock; see Fig. 1a, b). The sample was processed following a protocol described in detail in Stec et al. (2015). Isolated specimens were divided into four groups, destined for different analyses: (i) imaging of entire individuals by light microscopy (external and internal morphology and morphometry; 35 specimens), (ii) imaging of entire individuals by scanning electron microscopy (SEM, fine external morphology; 5 specimens), (iii) buccopharyngeal apparatus extraction and imaging by SEM (fine morphology of the apparatus; 4 specimens), and (iv) DNA extraction (including new molecular data for *Pilatobius*; 4 specimens).

Additionally, we examined by light microscopy four paratypes of *P. secchii* mounted in polyvinyl lactophenol, kindly loaned to us by Lorena Rebecchi (University of Modena and Reggio Emilia, Italy).

Microscopy and imaging

Specimens for light microscopy and morphometry were mounted on microscope slides in a small drop of Hoyer's medium prepared according to Morek et al. (2016) and examined under a Nikon Eclipse 50i phase-contrast microscope (PCM) associated with a Nikon Digital Sight DS-L2 digital camera. Specimens for SEM imaging were prepared according to Stec et al. (2015) and examined under high vacuum in a Versa 3D DualBeam SEM at the ATOMIN facility of Jagiellonian University. Buccopharyngeal apparatuses were extracted following a protocol by Eibye-Jacobsen (2001) with modifications described thoroughly in Gąsiorek et al. (2016), and examined under high vacuum in a Versa 3D DualBeam SEM at the ATOMIN facility of Jagiellonian University. All figures were assembled in Corel Photo-Paint X6 (version 16.4.1.1281). For deep structures that could not be fully focused in a single photograph, a series of 2–18 images were taken every ca. 0.2 µm then assembled into a single deep-focus image (using Corel).

Morphometrics

Sample size for morphometrics was chosen following recommendations by Stec et al. (2016). All measurements are given in micrometers (µm) and were performed under PCM using Nikon Digital Sight DS-L2 software. Structures

Fig. 1 Study area: **a** Svalbard Archipelago; **b** northern Spitsbergen and nearby islands, with Fuglesangen in a close-up (*inset*). *Scale bars* in km

were measured only if their orientations were suitable. Body length was measured from the anterior to the posterior end of the body, excluding the hind legs. Macroplacoid length sequence is given according to Kaczmarek et al. (2014). Claws were measured following Beasley et al. (2008). Buccopharyngeal tubes were measured following Pilato and Binda (1999). The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube, expressed as a percentage (Pilato 1981), presented herein in italics. Morphometric data were handled using the "Parachela" version 1.2 template available from the Tardigrada Register (Michalczyk and Kaczmarek 2013).

Establishing the neotype series

Given that the vast majority of the Richters' collection no longer exists (Dastych 1991), we designated all examined individuals as the neotype series.

Genotyping

DNA was extracted from individual animals following a Chelex® 100 resin (Bio-Rad) extraction method by Casquet et al. (2012) with modifications described in detail in Stec et al. (2015). We attempted to sequence four DNA fragments differing in effective mutation rate: the small and large ribosome subunit (18S rRNA and 28S rRNA, respectively), nuclear markers typically used for phylogenetic inference at high taxonomic level (e.g., Bertolani et al. 2014); the internal transcribed spacer (ITS-2), a noncoding nuclear fragment with high evolution rate, suitable for intraspecific comparisons as well as comparisons between closely related species (e.g., Gąsiorek et al. 2016); and finally, the cytochrome oxidase subunit I (COI), a protein-coding mitochondrial marker, widely used as a standard barcode gene with intermediate effective mutation rate (e.g., Bertolani et al. 2011). All fragments were amplified and sequenced according to the protocols described in Stec et al. (2015); primers and original references for specific polymerase chain reaction (PCR) programs are listed in Table 1. Sequencing products were read with the ABI 3130xl sequencer at the Molecular Ecology Lab, Institute of Environmental Sciences of Jagiellonian University, Kraków, Poland. Sequences were processed in BioEdit version 7.2.5 (Hall 1997).

Genetic distances

Given that our 28S rRNA, ITS-2, and COI sequences are the first molecular data for the subfamily Pilatobiinae, we could use only the 18S rRNA marker for genetic delineation of *P. recamieri*. We used all published 18S rRNA sequences for *Pilatobius* available from GenBank, i.e., *Pilatobius nodulosus* (Ramazzotti, 1957) HQ604934, *Pilatobius patanei* (Binda and Pilato, 1971) HQ604935–6, and *Pilatobius ramazzottii* (Robotti, 1970) HQ604939 (all by Bertolani et al. 2014). Sequences were aligned using the ClustalW Multiple Alignment tool (Thompson et al. 1994)

Table 1 Primers and references for specific protocols for amplification of the four DNA fragments sequenced in the study

DNA fragment	Primer name	Primer direction	Primer sequence (5′–3′)	Primer source	PCR program[a]
18S rRNA	SSU01_F	Forward	AACCTGGTTGATCCTGCCAGT	Sands et al. (2008)	Zeller (2010)
	SSU82_R	Reverse	TGATCCTTCTGCAGGTTCACCTAC	Sands et al. (2008)	
28S rRNA	28SF0001	Forward	ACCCVCYNAATTTAAGCATAT	Mironov et al. (2012)	Mironov et al. (2012)
	28SR0990	Reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov et al. (2012)	
COI	LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)	Michalczyk et al. (2012)
	HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)	
ITS-2	ITS3	Forward	GCATCGATGAAGAACGCAGC	White et al. (1990)	Wełnicz et al. (2011)
	ITS4	Reverse	TCCTCCGCTTATTGATATGC	White et al. (1990)	

[a] All PCR programs are also provided in Stec et al. (2015)

implemented in BioEdit. The aligned sequences were then trimmed to 777 bp. Uncorrected pairwise distances were calculated using MEGA6 (Tamura et al. 2013).

Data repository

The raw data underlying the redescription of *P. recamieri* are deposited in the Tardigrada Register (Michalczyk and Kaczmarek 2013) under www.tardigrada.net/register/0036.htm. DNA sequences were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank; accession numbers KX347526–31).

Results

Taxonomic account of the species

Phylum: Tardigrada Doyère, 1840

Class: Eutardigrada Richters, 1926

Order: Parachela Schuster, Nelson, Grigarick and Christensen, 1980

Superfamily: Hypsibioidea Pilato, 1969 (in Marley et al. 2011)

Family: Hypsibiidae Pilato, 1969

Subfamily: Pilatobiinae Bertolani, Guidetti, Marchioro, Altiero, Rebecchi and Cesari, 2014

Genus: *Pilatobius* Bertolani, Guidetti, Marchioro, Altiero, Rebecchi and Cesari, 2014

Pilatobius recamieri (Richters, 1911)

Diphascon recamieri; *terra typica*: Spitsbergen, Advent Fjord (ca. 78°14′N, 15°36′E), Hopen (ca. 76°34′N, 25°13′E); Richters (1911)

Hypsibius (*Diphascon*) *recamieri*; Spitsbergen; Marcus (1936)

H. (*D.*) *recamieri*; Spitsbergen, Hornsund Fjord: Ariekammen (ca. 77°00′N, 15°32′E), Rotjesfjellet (ca. 77°00′N; 15°24′E), Torbjørnsenfjellet (ca. 77°02′N, 15°18′E); Węglarska (1965)

D. recamieri; Spitsbergen, Hornsund Fjord: Tsjebysjovfjellet (ca. 76°56′N, 15°59′E) and Ariekammen, Albert I Land—Björnbukta (ca. 79°39′N, 12°24′E), Ny Friesland—Sör Glacier, Åsryggen (ca. 78°54′N; 18°01′E), Atomfjella—Tryggve Glacier (ca. 79°07′N, 16°42′E), Bünsow Land—Ebba Valley (ca. 78°42′N; 16°43′E) and Ebba Glacier (ca. 78°42′N; 16°47′E); Dastych (1985)

D. recamieri; Spitsbergen, Hornsund Fjord: Hyrne Glacier (77°03′N, 16°14′E); De Smet and Van Rompu (1994)

D. recamieri; Spitsbergen, Isbjörnhamna (vicinity of Polish Polar Station, ca. 77°00′N, 15°33′E); Janiec (1996)

D. recamieri; Spitsbergen, Ny Ålesund (ca. 78°55′N, 11°54′E) and Hornsund Fjord (Skrål Pynten); Maucci (1996)

D. recamieri; Hopen; Van Rompu and De Smet (1996)

D. recamieri; Spitsbergen, Rev Valley (ca. 77°01′N, 15°23′E) and Rotjesfjellet (ca. 77°00′N; 15°23′E); Kaczmarek et al. (2012)

D. recamieri; Spitsbergen, Ariekammen (77°00′N, 15°32′E); Zawierucha (2013)

D. recamieri; Prins Karls Forland (78°06′N, 14°51′E) and Edgeøya (78°53′N; 10°28′E); Zawierucha et al. (2013)

Material examined: Neotype and 39 neoparatypes from Fuglesangen, Svalbard, Norway (*terra typica*). Neotype and 26 neoparatypes (neotype and 21 neoparatypes on slides NO.022.01–4, and 5 neoparatypes on SEM stubs) are deposited together with extracted buccopharyngeal apparatuses in the Institute of Zoology and Biomedical Research, Jagiellonian University, Kraków, Poland; 13 neoparatypes (slides SV. Fug 00.02/1–3, 5–6) are deposited in the Department of Animal Taxonomy and Ecology,

Institute of Environmental Biology, Adam Mickiewicz University in Poznań, Poland.

Integrative redescription

Animals (see Table 2 for measurements): Body elongated, whitish, covered with smooth cuticle (Fig. 2a). Eyes usually present in live animals, but weakly developed or even absent in some specimens (Fig. 2a). Buccopharyngeal apparatus strongly elongated (Fig. 2b, c). The oral cavity armature, visible only under SEM, consists of 4–5 rows of minute conical teeth located in the rear of the oral cavity (Fig. 3a). Two distinct porous areas on the lateral sides of the crown visible by SEM only. Stylet furcae of the *Hypsibius* type (Fig. 2c; see Pilato and Binda 2010 for definitions of furca types). A prominent, dorsally placed, oval drop-like thickening on the border between the buccal and the pharyngeal tube present (Figs. 2b, c, 3b, c). Annulation regular, singular dorsally and ventrally, and net-like laterally; i.e., dorsal rings fork on the lateral walls of the tube and join with the neighboring forks into single rings that fork again into ventral rings, creating an interconnected network of thickenings (Figs. 3d, e). A short, very posterior part of the pharyngeal tube without annulation (Fig. 2c, arrowhead). Bulbus with two macroplacoids and a septulum (Fig. 2b). Macroplacoid length sequence $2 < 1$; macroplacoids bar-shaped, arranged diagonally (i.e., forming a rhomb). The first macroplacoid with an evident mid-constriction (Fig. 3f), in some specimens the constriction being so strong that the placoid may seem to be divided into two parts (Fig. 3g). The second macroplacoid with a slight subterminal constriction (Fig. 3g). An obvious drop-shaped or round septulum present (Fig. 3f, g). Claws of the *Hypsibius* type, with widened bases and with apparent accessory points on the primary branches (Fig. 4). Internal and anterior claws with two clear septa dividing the claw into the basal portion, the secondary branch, and the primary branch (Fig. 4a, b). External and posterior claws without septa. The base of the posterior claw extends towards the base of the anterior claw, forming a small cuticular bar (Fig. 4b, d, arrowhead). Anterior claws with pseudolunulae at their bases (Fig. 4b, d, empty arrow); pseudolunulae also sometimes weakly visible at the bases of internal claws I–III. External and posterior claws without pseudolunulae. No cuticular bars on legs I–III present.

Eggs: Roundish and smooth, deposited in exuviae (up to eight per clutch).

Molecular markers: The sequences for all four DNA markers were of good quality, however for a single individual the 28S rRNA fragment did not aplifyl. The sequenced fragments were of the following length: 1732 bp (18S rRNA; KX347526), 447 bp (28S rRNA; KX347527), 481 bp (ITS-2; KX347528), and 664 bp (COI; KX347529–31). The nuclear markers were represented by a single haplotype, whereas COI exhibited three haplotypes, all with minor *p*-distances between them (0.3–0.9%). The *p*-distances between the 18S haplotypes of all available *Pilatobius* species and *P. recamieri* varied between 1.2% (*P. patanei*) and 2.3% (*P. ramazzottii*), with the average distance of 1.7% (Table 3).

Etymology: Richters (1911) named the species after Joseph Récamier, a French zoologist.

Discussion

Comparison with earlier descriptions of *P. recamieri*

The original description by Richters (1911) is extremely limited and mentions only that animals can be up to 416 µm long, they are equipped with eyes, the buccal tube is approximately 2 µm wide, there are two macroplacoids in the bulbus, of which the first is longer than the second, and there is also an additional small structure posterior to the macroplacoids, termed "*virgule*," i.e., "a comma," meaning either a microplacoid or a septulum. All these traits are indeed present in the neotype population, but alone are not sufficient to differentiate *P. recamieri* from other *Pilatobius* species. Twenty-five years later, Marcus (1936) analyzed specimens from Spitsbergen and noted that they have a microplacoid and that *P. recamieri* can be differentiated from *P. oculatus* (Murray, 1906) by the length of the pharyngeal tube (which is longer in *P. recamieri*), and internal claw morphology. However, pharyngeal tubes in the two species can be of an almost equal length in specimens of a similar body size (pers. obs. based on an analysis of numerous individuals from Norway, Poland, and Scotland), and "internal claw morphology" is not a meaningful trait if not accompanied by more detail. In a faunistic survey, Węglarska (1959) described a population of *P. recamieri* from Southern Poland, in which some specimens had a septulum, whereas others a microplacoid. However, according to current knowledge on the morphological variability in eutardigrades, traits such as microplacoids and septulae are constant within species (e.g., Pilato 1975, 1981; Kosztyła et al. 2016). Therefore, either Węglarska (1959) found two similar *Pilatobius* species in one locality (but see also below) or her observation was erroneous. Given that, in some specimens, the septulum may be slightly crooked or twisted (e.g., compare Fig. 3f, g), it can be mistaken for a microplacoid. Ramazzotti and Maucci (1983) stated that *P. recamieri* can be distinguished from *P. oculatus* by the bulbus shape (less versus more spherical, respectively) and by the similarity of the external and internal claw shape (claws of a

Table 2 Measurements (in μm) of selected morphological structures of representatives of *Pilatobius recamieri* (Richters, 1911) mounted in Hoyer's medium

Character	*N*	Range		Mean		SD		Neotype	
		μm	*pt*	μm	*pt*	μm	*pt*	μm	*pt*
Body length	27	205–406	*1030–1625*	306	*1299*	49	*160*	322	*1425*
Buccopharyngeal tube									
Buccal tube length	30	19.8–28.2	–	23.6	–	1.8	–	22.6	–
Pharyngeal tube length	30	35.3–56.8	*175.6–231.3*	47.5	*200.9*	5.1	*13.5*	47.5	*210.2*
Buccopharyngeal tube length	30	55.4–85.0	*275.6–331.3*	71.1	*300.9*	6.6	*13.5*	70.1	*310.2*
Buccal/pharyngeal tube length ratio	30	43–57%	–	50%	–	3%	–	48%	–
Stylet support insertion point	30	12.9–18.0	*62.8–68.8*	15.6	*66.1*	1.2	*1.3*	15.2	*67.3*
Buccal tube external width	30	1.6–2.5	*6.6–10.0*	2.0	*8.7*	0.2	*0.7*	1.8	*8.0*
Buccal tube internal width	29	0.5–1.2	*2.5–4.7*	0.9	*3.7*	0.2	*0.5*	0.9	*4.0*
Placoid lengths									
Macroplacoid 1	30	3.9–7.3	*18.8–29.2*	5.6	*23.5*	0.8	*2.3*	5.2	*23.0*
Macroplacoid 2	30	2.5–5.5	*12.1–21.6*	4.0	*16.8*	0.7	*2.2*	3.6	*15.9*
Septulum	30	2.0–3.3	*9.2–14.0*	2.6	*11.2*	0.3	*1.0*	2.5	*11.1*
Macroplacoid row	30	6.9–13.7	*33.3–54.8*	10.5	*44.4*	1.6	*4.8*	10.0	*44.2*
Claw 1 lengths									
External base	17	2.7–5.6	*11.0–20.8*	4.0	*16.6*	0.8	*2.8*	3.3	*14.6*
External primary branch	14	5.2–10.1	*22.9–40.4*	8.0	*32.8*	1.3	*4.6*	8.2	*36.3*
External secondary branch	17	3.6–6.6	*15.7–26.4*	5.4	*22.2*	0.8	*2.8*	5.5	*24.3*
Internal base	14	2.4–4.6	*10.5–19.5*	3.8	*15.9*	0.7	*2.8*	2.4	*10.6*
Internal primary branch	11	4.3–6.8	*18.1–28.0*	6.0	*24.4*	0.7	*2.7*	5.8	*25.7*
Internal secondary branch	13	3.0–4.9	*13.0–20.8*	3.9	*16.3*	0.7	*2.2*	?	*?*
Claw 2 lengths									
External base	27	2.9–6.5	*14.6–27.2*	4.5	*19.0*	1.0	*3.5*	4.2	*18.6*
External primary branch	27	5.0–11.8	*21.0–45.6*	9.0	*38.2*	1.8	*5.8*	9.7	*42.9*
External secondary branch	27	3.6–7.3	*17.6–36.1*	5.8	*24.4*	1.1	*3.8*	4.8	*21.2*
Internal base	19	2.2–4.9	*10.6–21.4*	3.9	*16.6*	0.9	*3.3*	3.5	*15.5*
Internal primary branch	18	4.4–9.1	*19.6–36.4*	6.7	*28.0*	1.3	*4.5*	6.9	*30.5*
Internal secondary branch	19	3.5–6.2	*16.1–24.8*	4.6	*19.6*	0.8	*2.5*	5.4	*23.9*
Claw 3 lengths									
External base	27	2.8–6.0	*12.2–25.9*	4.5	*18.8*	0.9	*3.2*	3.9	*17.3*
External primary branch	26	7.1–11.5	*35.3–45.6*	9.9	*41.4*	1.0	*2.8*	9.9	*43.8*
External secondary branch	26	4.1–7.2	*18.9–29.8*	5.9	*24.7*	0.8	*2.6*	5.5	*24.3*
Internal base	22	2.7–5.4	*11.8–23.2*	4.1	*17.4*	0.9	*3.3*	3.3	*14.6*
Internal primary branch	18	4.3–9.6	*19.9–38.4*	6.7	*28.7*	1.4	*5.0*	?	*?*
Internal secondary branch	20	3.5–5.8	*15.6–23.6*	4.7	*20.2*	0.6	*2.1*	5.2	*23.0*
Claw 4 lengths									
Anterior base	21	2.6–5.4	*12.9–21.1*	4.3	*18.5*	0.8	*2.5*	3.6	*15.9*
Anterior primary branch	20	4.4–9.1	*21.7–36.4*	6.6	*28.3*	1.3	*3.8*	6.7	*29.6*
Anterior secondary branch	21	3.3–5.6	*15.6–24.8*	4.5	*19.1*	0.7	*2.3*	5.6	*24.8*
Posterior base	25	2.8–6.0	*13.9–25.0*	4.7	*20.1*	0.8	*2.6*	4.3	*19.0*
Posterior primary branch	23	7.1–13.6	*34.3–54.4*	10.8	*45.2*	1.7	*5.0*	10.6	*46.9*
Posterior secondary branch	25	3.9–7.5	*19.4–29.2*	6.0	*25.6*	1.0	*2.7*	6.3	*27.9*

N number of specimens/structures measured, "Range" indicates the smallest and largest structure among all measured specimens, *SD* standard deviation

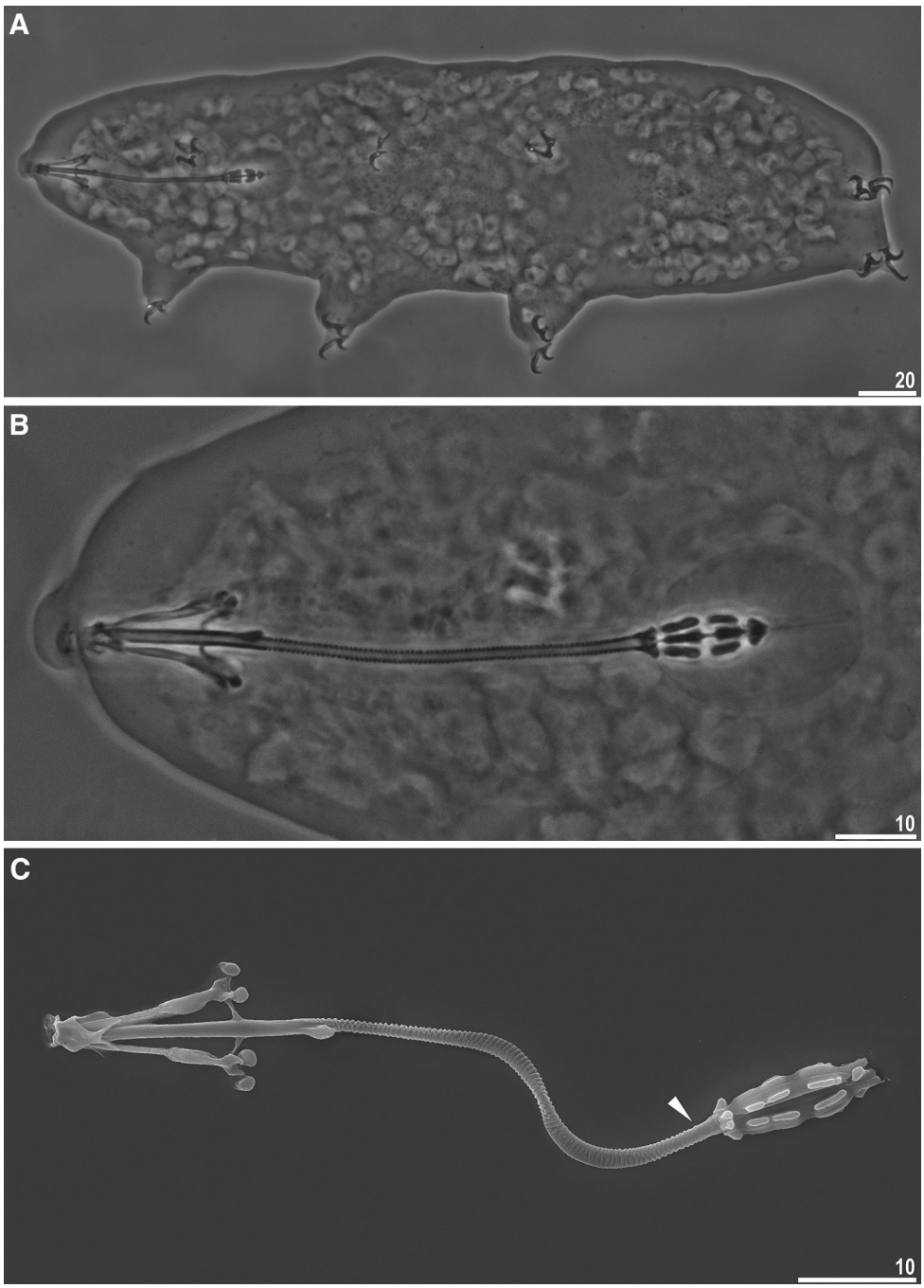

Fig. 2 *Pilatobius recamieri* (Richters, 1911): **a** habitus, lateroventral view (PCM, neotype); **b**, **c** buccopharyngeal apparatus, dorsolateral view: **b** PCM (neotype), **c** SEM; *arrowhead* indicates the nonannulated posterior portion of the pharyngeal tube (neoparatype). *Scale bars* in µm

dissimilar shape in *P. oculatus*). Although it is now recognized that the bulbus is prone to deformation under cover slip pressure and slight differences in bulbus shape should not be used for species differentiation (Pilato 1981), the second trait seems valid. The most comprehensive record of *P. recamieri* to date was by Dastych (1985), who analyzed numerous specimens from different parts of Spitsbergen (i.e., the *terra typica*). He noted that the first macroplacoid is 1.2–1.4 times longer than the second one (which is consistent with our measurements, see Table 2), and that the majority of individuals have a small thickening at the end of the second macroplacoid (also present in almost all individuals from the neotype series). Unfortunately, Dastych (1985) did not address the microplacoid versus septulum issue, although he described the thin cuticular extension of the bases of the posterior claws that we also show in the present study (Fig. 3b, d, arrowhead). In the most recent description of *P. recamieri*, based on specimens collected from Poland, Dastych (1988) did note the microplacoid. Thus, similarly to the earlier record by

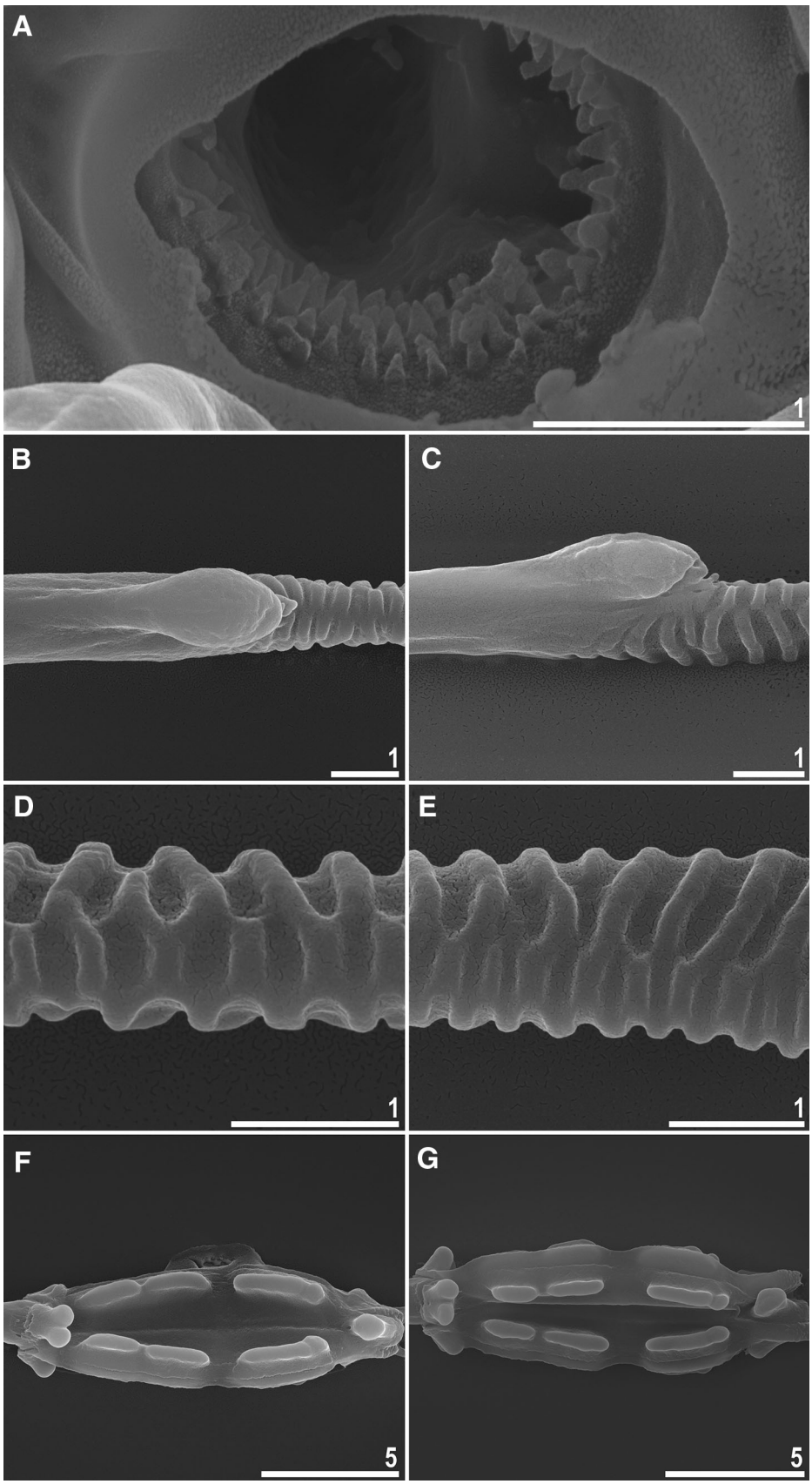

Fig. 3 *Pilatobius recamieri* (Richters, 1911), details of the buccopharyngeal apparatus (all neoparatypes in SEM): **a** oral cavity armature; **b**, **c** drop-like thickening on the buccopharyngeal tube (dorsal and lateral view, respectively); **d** annulation of the anterior portion of the pharyngeal tube (lateral view); **e** annulation of the posterior portion of pharyngeal tube (dorsolateral view); **f** a typical pharyngeal structures; **g** a slightly aberrant pharyngeal structures in which the septulum is crooked and resembles a microplacoid. *Scale bars* in μm

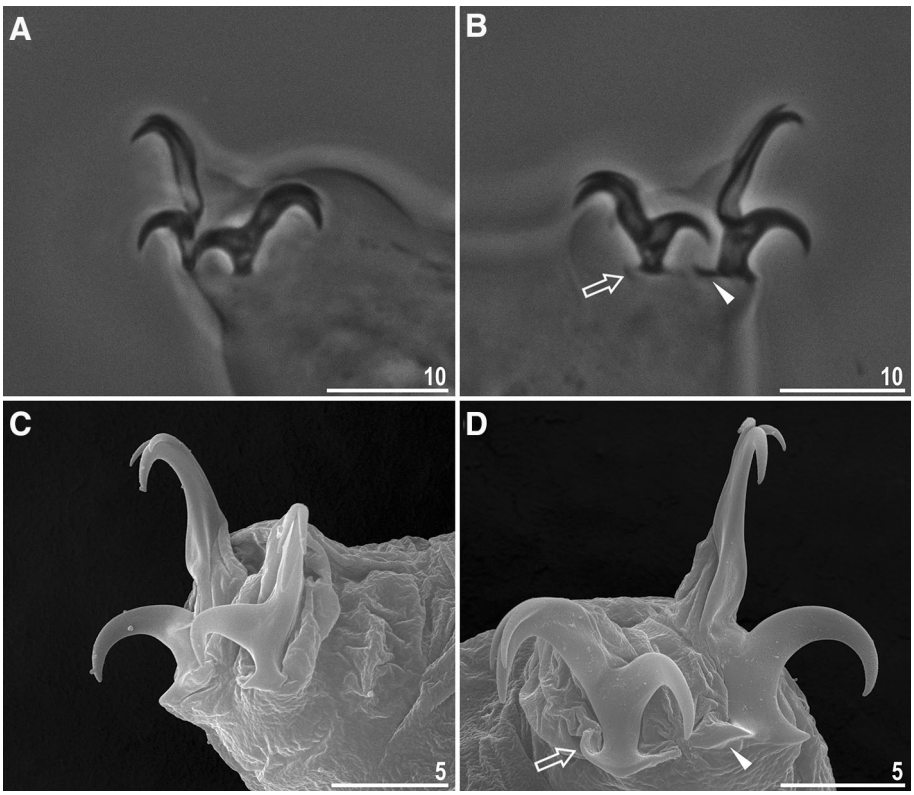

Fig. 4 *Pilatobius recamieri* (Richters, 1911), claws: **a**, **b** claws III and IV, respectively, seen in PCM; *empty arrow* points at the pseudolunula under the anterior claw base, *arrowhead* indicates the small cuticular bar at the posterior claw base (neotype); **c**, **d** claws III and IV, respectively, seen in SEM; *empty arrow* points at the pseudolunula under the anterior claw base, *arrowhead* indicates the cuticular bar protruding towards the pseudolunula (neoparatype). *Scale bars* in μm

Węglarska (1959), either his record did not represent *P. recamieri* but a similar species or he misinterpreted the septulum as the microplacoid.

Differential diagnosis

The great majority of currently known *Pilatobius* species exhibit a sculptured cuticle. Apart from *P. recamieri*, there are only three other congeners with smooth cuticle: *P. brevipes* (Marcus, 1936), *P. borealis* (Biserov, 1996), and *P. secchii* (Bertolani and Rebecchi, 1996). Although *P. oculatus* (Murray, 1906) has a sculptured cuticle, it can be misidentified as *P. recamieri* given that the cuticular sculpturing in the former species, composed of small polygons limited to the caudal cuticle, may sometimes be poorly visible under low magnification.

Of all *Pilatobius* species, *P. recamieri* is definitely most similar to *P. secchii*. At the time of description of *P. secchii*, no detailed information on the morphology of *P. recamieri* was available and Bertolani and Rebecchi (1996) assumed that the cuticular bar on hind legs and pseudolunulae under internal and anterior claws were characteristic for *P. secchii* and absent in *P. recamieri*. However, we have now shown that these traits are, in fact, present in *P. recamieri* (see Fig. 3), meaning that the two species are morphologically indistinguishable. Thus, morphometric or/and molecular data are needed to test whether *P. secchii* is a valid species or a synonym of *P. recamieri*. Unfortunately, Bertolani and Rebecchi (1996) did not provide a complete set of morphometric data, and the limited measurements available for the holotype do not allow a confident differentiation of the two species. Thus, we measured four *P. secchii* paratypes, kindly loaned to us by Lorena Rebecchi, according to modern morphometric standards. We found that the ranges for all 71 absolute and relative traits of *P. secchii* overlap with those for *P. recamieri* (compare Tables 2, 4). Importantly, Bertolani and Rebecchi (1996) reported a higher *pt* value for the stylet support insertion point compared with our measurements (*73.9 %* versus *63.6–66.0 %*, respectively). If the *pt* value provided by Bertolani and Rebecchi (1996) is true, then it would constitute a clear morphometric difference between these two taxa. However, the higher *pt* reported by Bertolani and Rebecchi (1996) is most likely a result of a

Table 3 *p*-Distances (in %) between all currently published *Pilatobius* spp. 18S rRNA sequences

Species (sequence)	1	2	3	4
1. *P. nodulosus* (HQ604934)				
2. *P. patanei* #1 (HQ604935)	2.8			
3. *P. patanei* #2 (HQ604936)	2.7	0.1		
4. *P. ramazzottii* (HQ604939)	2.3	1.8	1.7	
5. *P. recamieri* (KX347526)	2.1	1.3	1.2	2.3

Table 4 Measurements (in μm) of selected morphological structures of four paratypes of *Pilatobius secchii* (Bertolani and Rebecchi, 1996) mounted in polyvinyl lactophenol

Character	*N*	Range		Mean		SD	
		μm	*pt*	μm	*pt*	μm	*pt*
Body length	4	149–277	*756–1365*	214	*1025*	52	*253*
Buccopharyngeal tube							
Buccal tube length	4	19.7–22.0	–	20.8	–	1.0	–
Pharyngeal tube length	2	33.1–47.6	*150.5–234.5*	40.4	*192.5*	10.3	*59.4*
Buccopharyngeal tube length	2	55.1–67.9	*250.5–334.5*	61.5	*292.5*	9.1	*59.4*
Buccal/pharyngeal tube length ratio	2	43–66 %	–	55 %	–	17 %	–
Stylet support insertion point	4	13.0–14.0	*63.6–66.0*	13.5	*64.8*	0.6	*1.2*
Buccal tube external width	4	1.7–1.9	*8.0–9.4*	1.8	*8.8*	0.1	*0.6*
Buccal tube internal width	4	0.9–1.3	*4.6–6.4*	1.1	*5.3*	0.2	*0.8*
Placoid lengths							
Macroplacoid 1	4	4.8–5.9	*24.4–29.1*	5.5	*26.2*	0.5	*2.1*
Macroplacoid 2	4	3.3–4.2	*16.8–20.2*	3.9	*18.6*	0.4	*1.4*
Septulum	4	1.9–2.8	*9.4–12.7*	2.2	*10.4*	0.4	*1.6*
Macroplacoid row	4	8.6–11.3	*43.7–51.4*	10.1	*48.4*	1.1	*3.3*
Claw 1 lengths							
External base	4	2.5–3.1	*11.7–14.8*	2.8	*13.3*	0.3	*1.4*
External primary branch	1	?	*?*	6.5	*30.5*	?	*?*
External secondary branch	4	3.8–4.7	*17.7–23.2*	4.2	*20.0*	0.4	*2.3*
Internal base	2	2.6–3.0	*13.2–14.8*	2.8	*14.0*	0.3	*1.1*
Internal primary branch	0	?	*?*	?	*?*	?	*?*
Internal secondary branch	0	?	*?*	?	*?*	?	*?*
Claw 2 lengths							
External base	4	3.0–4.2	*14.1–20.7*	3.6	*17.2*	0.5	*2.7*
External primary branch	2	7.7–8.6	*36.2–42.4*	8.2	*39.3*	0.6	*4.4*
External secondary branch	4	4.5–5.1	*21.1–25.1*	4.8	*22.9*	0.3	*1.9*
Internal base	4	2.7–4.1	*13.1–20.2*	3.2	*15.3*	0.6	*3.3*
Internal primary branch	2	5.3–5.9	*26.9–29.1*	5.6	*28.0*	0.4	*1.5*
Internal secondary branch	1	?	*?*	4.6	*22.7*	?	*?*
Claw 3 lengths							
External base	4	3.3–4.6	*16.8–22.7*	4.0	*19.2*	0.6	*2.7*
External primary branch	2	7.5–7.6	*35.7–36.9*	7.6	*36.3*	0.1	*0.9*
External secondary branch	4	4.7–5.4	*23.0–25.6*	5.1	*24.3*	0.3	*1.1*
Internal base	3	3.7–3.9	*16.8–19.2*	3.8	*17.8*	0.1	*1.3*
Internal primary branch	2	5.5–5.7	*25.8–28.1*	5.6	*27.0*	0.1	*1.6*
Internal secondary branch	3	3.8–4.5	*17.8–21.2*	4.2	*19.8*	0.4	*1.8*
Claw 4 lengths							
Anterior base	4	3.4–4.4	*17.3–20.0*	3.9	*18.6*	0.4	*1.5*
Anterior primary branch	3	5.0–5.2	*22.7–26.4*	5.1	*24.9*	0.1	*1.9*
Anterior secondary branch	2	3.7–4.3	*18.8–21.2*	4.0	*20.0*	0.4	*1.7*
Posterior base	4	4.2–4.9	*20.0–24.1*	4.6	*21.9*	0.3	*1.7*
Posterior primary branch	4	7.3–8.5	*33.2–39.9*	7.8	*37.7*	0.5	*3.1*
Posterior secondary branch	4	5.3–5.7	*24.9–27.4*	5.5	*26.3*	0.2	*1.2*

N number of specimens/structures measured, "Range" indicates the smallest and largest structure among all measured specimens, *SD* standard deviation

different method of buccal tube measurement compared to that adopted by us. Three years after the description of *P. secchii*, Pilato and Binda (1999) proposed that, in taxa with a drop-like thickening, the buccal tube length should be measured down to the posterior end of the drop. Previously, some authors measured the buccal tube length to the anterior end of the drop, which translates to a shorter measurement and ultimately to overestimated *pt* values. In other words, currently there are no morphological or morphometric differences between *P. recamieri* and *P. secchii* and a molecular investigation is needed to verify the status of the latter species. Therefore, taking into account the evidence described above, we must designate *P. secchii* as *nomen inquirendum* until DNA sequences become available for this taxon.

In contrast to *P. secchii*, *P. recamieri* is readily distinguishable from the other three above-mentioned species and differs specifically from:

- *P. brevipes*, known from various localities in the Palearctic and from some Nearctic habitats (Ramazzotti and Maucci 1983), by the lack of bars under claws I–III and by a longer pharyngeal tube (35.3–56.8 µm in 205–406-µm-long specimens of *P. recamieri* versus around 30 µm in a 350-µm-long specimen of *P. brevipes*). We must underline that this difference may result from the use of a different measurement technique, as in the case of *P. secchii* (see above).
- *P. borealis*, recorded only from the type locality in the sub-Arctic Taimyr Peninsula (Biserov, 1996), by a more anterior stylet support insertion point (*pt 62.8–68.8 %* in *P. recamieri* versus *68.8–73.3 %* in *P. borealis*), and a significantly longer buccal and pharyngeal tube (respectively 19.8–28.2 µm and 35.3–56.8 µm in *P. recamieri* versus 14–16 µm and 22–26 µm in *P. borealis*).
- *P. oculatus*, reported from various localities in the Holarctic (Ramazzotti and Maucci 1983), by the internal morphology of external primary branches (a uniform structure from base to tip in *P. recamieri* versus a light-refracting unit within the base of the branch) (Dastych 1988), and caudal polygonal sculpture (absent in *P. recamieri* versus present in *P. oculatus*).

Although Bertolani and Rebecchi (1996) described the structure under the anterior claw of *P. secchii* as the "lunula," we think that the term "pseudolunula" is more appropriate, since well-defined lunulae, present in numerous genera of the superfamilies Macrobiotoidea (Thulin 1928) and Eohypsibioidea (Bertolani & Kristensen 1987), are connected with the claw base via a peduncle, whereas the structures under claws in Hypsibioidea (Pilato 1969) and Isohypsibioidea (Sands et al. 2008) are placed directly at the claw base and are usually thin-edged, making them difficult to identify in some cases. In fact, macrobiotoid and eohypsibioid lunulae are always evident under SEM, whereas pseudolunulae in the hypsibioid and isohypsibioid taxa are never identifiable in SEM and appear as widened claw bases rather than separate structures (e.g., see Fig. 3).

Geographic distribution of the species

Pilatobius recamieri has been reported from a number of Holarctic localities, being found most often in mountainous habitats and only rarely in lowlands (Dastych 1988). It was recorded from various northern areas, such as the Svalbard Archipelago (Dastych 1985; Maucci 1996; Zawierucha et al. 2013), Greenland (Petersen 1951), Taimyr Peninsula and Novaya Zemlya (Biserov 1996, 1999), Vancouver Island (Kathman 1990), and Colorado (Beasley 1990). Another group of records constitute mountain populations (possibly postglacial relicts), e.g., Töw Province in Mongolia (Iharos 1965, 1968) or the Elburz Range in Iran (Dastych 1972). If *P. secchii* turns out to be synonymous with *P. recamieri*, the Tusco-Emilian Apennine population should be added to the mountainous reports of the species. There are also some lowland European records from eastern Italy (Durante Pasa and Maucci 1975) and southern Hungary (Vargha 1998). Finally, the species was reported from Southern Argentina (Iharos 1963; Rossi and Claps 1991), but these records are considered questionable and most likely belong to a different species, as hypothesized by Dastych (1988) and Kaczmarek et al. (2015). However, given the limited original description of *P. recamieri*, even the Holarctic records outside of the *terra typica* should be treated with caution and verified against the morphological and molecular data provided herein, as some of the more distant reports may belong to similar species rather than to *P. recamieri*. A recent study by Gąsiorek et al. (2016) suggested that *Mesocrista spitzbergensis*, another species originally described from Svalbard and later reported from numerous localities throughout the Holarctic, probably does not occur in Continental Europe. This was possible thanks to a thorough redescription of *M. spitzbergensis* from the type locality and an integrative comparison with several European populations of *Mesocrista* that revealed a new species (Gąsiorek et al. 2016). Therefore, it would not be surprising if at least some of the non-Arctic records of *P. recamieri* represented other *Pilatobius* species, only superficially similar to *P. recamieri*. Our integrative redescription makes such evaluations possible.

Conclusions

An integrative approach to the redescription of *P. recamieri* allowed us to establish three important morphological traits that characterize the species and that were uncertain or

unknown to date: the presence of the septulum instead of the microplacoid, the presence of pseudolunulae at the internal and anterior claw bases, and the presence of cuticular bars next to the bases of posterior claws. DNA sequencing provided neotype barcodes for species delimitation that will enable confident verification of future records of the species as well as phylogenetic analyses of the genus *Pilatobius*. Given that we found no morphological or morphometric differences between *P. recamieri* and *P. secchii*, a species described from Italy 85 years after *P. recamieri*, the taxonomic status of *P. secchii* must be considered uncertain, pending future verification contingent on molecular data for the type population. Because of the ambiguities concerning earlier *P. recamieri* records, no sound conclusions on the geographic range of the species can be drawn at the moment, but it is possible that *P. recamieri* is an Arctic or a Palaearctic/Holarctic mountainous species, meaning that the alleged lowland records and reports from other zoogeographic realms should be treated with caution.

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